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# New Approaches for the Preparation of Hydrophobic Heparin Derivatives

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**Abstract** □ A heparin derivative sufficiently lipophilic to be bound to plastics, forming blood-compatible supports, or to be used as an anticoagulant by transdermal or oral routes would be of great pharmaceutical interest. For such applications, the functional groups within heparin's antithrombin III binding site, responsible for its anticoagulant activity, cannot be modified. Chemistry is described in which lipophilic substituents were attached to the reducing termini of heparin chains. Substituents introduced at this position had a minimal effect on the antithrombin III binding sites found in heparin's interior. These derivatives, with enhanced hydrophobicities, were prepared using two distinctly different approaches. First, octyl isocyanate and octadecyl isocyanate were coupled to the core peptide of peptidoglycan heparin to form octyl- and octadecyl-peptidoglycan heparin. These octyl- and octadecyl-peptidoglycan heparins were then purified by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B, demonstrating their enhanced hydrophobicities. Second, the lipophilic acyl hydrazides of various long chain fatty acids were coupled to heparin's reducing end. Caprylic ($C_8$), capric ($C_{10}$), lauric ($C_{12}$), and stearic ($C_{18}$) hydrazide derivatives of heparin were prepared using this approach. Only the stearyl hydrazide derivative of heparin showed a measurable increase in lipophilicity. This result demonstrated that a single small linear $C_8$, $C_{10}$, or $C_{12}$ aliphatic chain was ineffective in enhancing the hydrophobicity of the highly negative, polyanionic heparin molecule. Two lipophilic chains, lauryl ($C_{12}$) and stearyl ($C_{18}$), were then coupled to a single heparin chain, resulting in a heparin derivative having enhanced hydrophobicity. All the heparin derivatives prepared in this study maintained some of their anticoagulant activity.

## Introduction

Heparin is a polydisperse, highly sulfated, linear polysaccharide composed of repeating 1→4 linked uronic acid and glucosamine residues.[1] Heparin is primarily used as an anticoagulant as it contains a specific pentasaccharide sequence that binds to and activates antithrombin III (ATIII), a coagulation protease inhibitor.[1,2] It is being investigated as an agent to regulate complement activity, angiogenesis, atherosclerosis, and viral activity and to stabilize and activate growth factors.[2] More than 500 million doses of heparin are used as an anticoagulant in the world each year.[2] Although efficacious by intravenous administration and to a lesser degree following subcutaneous administration, heparin is ineffective when administered orally or transdermally. The requirement that heparin be injected limits its application to hospitals and clinics and thus has impeded the development of new prophylactic applications. Heparin's high molecular weight (5000–25 000, average 14 000) and highly polar, polyanionic character are primarily responsible for its limited bioavailability. Low molecular weight (LMW) heparin has enhanced subcutaneous bioavailability, but its highly polar nature still limits its use by oral and transdermal routes.[2] Transdermal and oral adsorption of heparin and LMW heparin might, however, be enhanced through derivatization that increases lipophilicity.

Bârzu *et al.*[3] prepared LMW heparin by periodate depolymerization of heparin and modified these by *O*-acylation. Although these derivatives have low anticoagulant activity, they display a high activity toward smooth muscle cell antiproliferation. This activity was related to the degree of acylation. Heparin's duration of action and antithrombotic activity have also been enhanced without increasing its prohemorrhagic properties by preparing butylated LMW heparin.[4] Heparin has been derivatized by modification at carboxylate groups of the uronic acid residues, at hydroxyl groups on various sugar residues, and at amino groups afforded through de-*N*-acetylation or de-*N*-sulfation.[5] These derivatization reactions generally lack specificity, damage the ATIII binding site, and thus often result in reduction or loss of anticoagulant activity.

A second major application for heparin is to maintain the hemocompatibility of extracorporeal devices.[6] Such devices as heart lung oxygenators and kidney dialyzers are composed of a variety of different plastics. Heparin bonding to the surfaces of these plastics represents an effective approach of maintaining hemocompatability. Heparin has been depolymerized with nitrous acid to afford a low molecular weight heparin having a reactive aldehyde at its reducing end. This low molecular weight heparin has been covalently attached to amine-containing surfaces through reductive amination in an effort to prepare blood-compatible surfaces.[7] Hydrophobic heparin derivatives might be useful as a coating for such plastic surfaces. Again, these hydrophobic heparin derivatives must maintain their anticoagulant activity to prepare effective hemocompatible surfaces.

This paper describes two new approaches for preparing heparin derivatives, with slightly enhanced hydrophobicity that retain anticoagulant activity. Chemistry is developed by which lipophilic substituents of any length (resulting in the desired lipophilicity) could be attached to the reducing termini of heparin chains with a minimal effect on the ATIII binding sites found in the chain's interior.

## Experimental Section

**Materials**—Raw heparin (Stage 12) (150 IU/mg, containing 19 μmol of amino groups/g; determined by the trinitrobenzenesulfonate method[8]), bleached heparin (158 IU/mg), and chromogenic Xa substrate were obtained from Celsus Laboratories, Inc. (Cincinnati, OH). Bovine factor Xa was from Haemachem (St. Louis, MO). Octyl isocyanate, octadecyl isocyanate, ethyl caprylate, ethyl caproate, methyl laurate, palladium on activated charcoal, and tetrabutylammonium hydroxide were from Aldrich Chemical Co. (Milwaukee, WI). Fluoraldehyde protein/peptide assay reagent used in measuring the presence of free amino groups was from Pierce (Rockford, IL). Phenyl-Sepharose CL-4B and Dowex $H^+$ form (mesh, 50) were from Sigma Chemical Co. (St. Louis, MO). Ultrapure, electrophoresis-grade formamide was obtained from Boehringer (Indianapolis, IN). All other chemicals and reagents were from Sigma. Pressure filtration equipment and membranes were from Amicon (Beverly, MA). Dialysis membrane of 3500 molecular weight cutoff (MWCO) was from Spectrum (Houston, TX).

**Methods**—*Preparation of Octyl-Peptidoglycan Heparin*—Raw heparin (100 mg) was dissolved in 20 mL of formamide by shaking at 250 rpm for 10 h at 37 °C. Octyl isocyanate (1.5 μL) was added to this solution and shaken for an additional 12 h. The reaction mixture was then diluted 20-fold with distilled water and the volume was reduced to 20 mL by pressure filtration using a 3000 MWCO membrane. The

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concentrated solution was dialyzed for 3 days against water using a 3500 MWCO membrane and the octyl-peptidoglycan heparin was freeze-dried.

*Preparation of Octadecyl-Peptidoglycan Heparin*—Since octadecyl isocyanate was insoluble in formamide, a less polar organic solvent, chloroform, was selected. Heparin sodium salt was first converted to the chloroform soluble tetrabutylammonium salt. Raw heparin (100 mg/10 mL of water) was passed through a Dowex 50 ($H^+$ form) column of dimensions 1 × 10 cm. The column was washed with 20 mL of 30 mM hydrochloric acid and the wash was collected and neutralized to pH 4.5 with tetrabutylammonium hydroxide. The heparin tetrabutylammonium salt was recovered by freeze-drying. Tetrabutylammonium heparin (90 mg) was dissolved in 25 mL of chloroform and shaken for 10 h at 37 °C. Octadecyl isocyanate (2 mL) was added and the reaction was shaken for 24 at 37 °C. The reaction was extracted with 20 mL of 16% sodium chloride, several hours being allowed for the two phases to separate. The aqueous phase was recovered and the waxy precipitate was removed by centrifugation (15 min at 5000 rpm). The aqueous phase was then reduced to half its volume on a rotary evaporator to remove any residual chloroform and transferred to a dialysis bag (MWCO 3500). After exhaustive dialysis against water, the retentate was freeze-dried.

*Purification of Octyl- and Octadecyl-Peptidoglycan Heparin*—The procedure followed the method of Uchiyama[9] and Ogamo[10] with minor modifications. Octyl- or octadecyl-peptidoglycan heparin (100 mg) was dissolved in 5 mL of 3.8 M ammonium sulfate at pH 3.3 and loaded on a 20 × 1.5 cm phenyl-Sepharose CL-4B column prepared in the same solution. The column was washed at 4 °C with 200 mL of the same solution, and then with a reverse, stepwise gradient of 200 mL each of the following ammonium sulfate solutions: 3.8 M (pH 3.3), 3.4 M (pH 3.35), 3.0 M (pH 3.4), and 2.0 M (pH 3.5). Octyl-peptidoglycan heparin was then eluted from the column with water. Octadecyl-peptidoglycan heparin remained bound on washing the column with water and only eluted with 35% ethanol in water. A column flow rate of 20 mL/h was used and 3-mL fractions were collected. Elution was monitored by taking samples (0.2 mL) and analyzing by the carbazole reaction.[11] The column was regenerated by washing with an equal volume of 95% ethanol. The water (or ethanol water) fraction containing octyl- or octadecyl-peptidoglycan heparin was dialyzed (MWCO 3500) and freeze-dried. The 7-mg recovery of octyl-peptidoglycan heparin corresponded to a 7% isolated yield (based on raw heparin starting material). The octadecyl-peptidoglycan heparin was recovered in 3% yield.

*$^1H$ NMR Analysis of Octyl-Peptidoglycan Heparin*—The sample was dissolved in $^2H_2O$ (99.9%) and freeze-dried to exchange hydroxyl protons with deuterium. After exchanging three times, the sample was dissolved in 500 μL of $^2H_2O$ (99.99%) containing deuterated (trimethylsilyl)-propionic acid sodium salt (0.03% w/v) as internal reference. The $^1H$-NMR spectra were obtained using a Varian Unity 500 spectrometer at 500 MHz or a Bruker WM 360 spectrometer at 360 MHz at ambient temperature. The spectra at 360 MHz had the following assignments: residue IdoAp2S, δ 5.25 (H-1), 4.40 (H-2), 4.20 (H-3), 4.10 (H-4); residue GlcNp2S6S, δ 5.50 (H-1), 3.80 (H-2), 3.70 (H-3), 3.90 (H-4), 4.15 (H-5); residue GlcAp, δ 5.55 (H-1); residue GlcNAc, δ 2.20 (s, $COCH_3$); residue GlcNpAc, δ 2.25; methylene protons of alkyl chain resonated at δ 1.45–1.55 [m, $CH_3(CH_2)_nCH_2CO$], 1.60–1.70 [m, $CH_3(CH_2)_nCH_2CO$], 0.97 [br t, $CH_3(CH_2)_nCH_2CO$]. IdoAp2S is 2-*O*-sulfo-α-L-idopyranosyluronic acid; GlcNp2S is 2-*N*-sulfo-2-deoxy-2-amino-α-D-glucopyranose; GlcAp is β-D-glucopyranosyluronic acid; GlcNAc is *N*-acetyl-D-glucosamine.

*Preparation of $C_8$ and $C_{12}$ Hydrazides*—The procedure for the preparation of octyl, capryl, and lauryl hydrazides followed the method reported by Martinez.[12] For example, octyl hydrazide was prepared by stirring a solution of caprylic acid ethyl ester (0.13 mol in 100 mL of 95% ethanol) and 3.77 mL of anhydrous hydrazine (0.12 mol) for 18 h at room temperature. The reaction mixture was reduced to half the original volume by rotary evaporation and the reaction was kept at 4 °C until the octyl hydrazide crystallized. The octyl hydrazide showed a characteristic multiple band feature in the 3600–3700-$cm^{-1}$ region in the Fourier-transformed infrared (FT-IR) spectrum assignable to the hydrazide ($NHNH_2$) linkage. The octyl, capryl, and lauryl hydrazides are all liquid at 25 °C and were each recovered in yields of >70%.

*Preparation of the $C_8$, $C_{10}$, and $C_{12}$ Heparin Hydrazide Derivatives*—The three heparin hydrazide derivatives followed a common preparation procedure. For example, the octyl ($C_8$) hydrazide derivative was prepared by dissolving 7.1 mmol of heparin (the average molecular weight of heparin is 14 000) in 30 mL of formamide and incubating in a closed 50-mL plastic centrifuge tube on a shaker (250 rpm) at 37 °C for 10 h. Octyl hydrazide (71 mmol) dissolved in 1 mL of *N,N*-dimethylformamide was added and the solution shaken for an additional 12 h. The solution was diluted 20-fold with water and then concentrated by pressure filtration using a 3000 MWCO membrane. The concentrated sample was dialyzed in 3500 MWCO bags against water for 3 days and freeze-dried.

*Purification of the $C_8$, $C_{10}$, $C_{12}$ Heparin Hydrazide Derivatives*—The dry heparin hydrazide derivative (30 mg) was dissolved in ca. 1 mL of 16% sodium chloride solution and precipitated by adding 70% methanol.[13] The suspension was kept overnight at 4 °C, to ensure all the heparin hydrazide derivative had precipitated, after which the precipitate was recovered by centrifugation at 2000 rpm for 10 min. Finally, the heparin hydrazide derivative was reconstituted in water, dialyzed against water using a 3500 MWCO membrane, and freeze-dried. The recovered yield was between 36 and 76% for the heparin hydrazide derivatives.

*Preparation of Stearyl ($C_{18}$) Hydrazide*—A solution of 3.52 mmol of stearic acid in 50 mL of absolute ethanol and 3 mL of concentrated sulfuric acid was refluxed for 12 h. The reaction mixture then neutralized with 10% aqueous sodium bicarbonate and the solution was extracted with methylene chloride. The organic phase containing ethyl stearate was dried with anhydrous sodium sulfate and evaporated. Ethyl stearate (0.852 g) in 30 mL of ethanol was placed in a round-bottom flask and 3 mL of anhydrous hydrazine was added dropwise. The reaction mixture was stirred at 45 °C for 4 h and cooled to room temperature. Unreacted hydrazine was evaporated under vacuum. The stearyl hydrazide obtained was recrystallized from absolute ethanol (83% yield). The stearyl hydrazide gave the expected infrared spectrum and had a mp 102–105 °C.

*Preparation of the Stearyl Hydrazide Heparin Derivative*—The sodium salt of heparin was first transformed into tetrabutylammonium salt. A minicolumn was packed with 3 mL of Dowex 50 ($H^+$ form) and washed with excess 50% aqueous tetrabutylammonium hydroxide to transform it to the $Bu_4N^+$ form. The column was next washed thoroughly with distilled water until the pH of the washes were neutral. A heparin solution (26.0 mg/3 mL of water) was passed through Dowex ($Bu_4N^+$ form) cation-exchange resin, and the column was rinsed with two volumes of water. The combined eluent and washes, containing the tetrabutylammonium salt of heparin, was dialyzed and freeze-dried. The tetrabutylammonium salt of heparin (0.7 nmol) in 25 mL of dry methylene chloride was stirred with 0.325 mM stearyl hydrazide at 45 °C for 24 h. The methylene chloride was evaporated to dryness and the residue dissolved in 5 mL of water, filtered through Whatman #1 filter paper, and passed through Dowex 50 ($Na^+$ form), and after washing of the column with water, the sodium salt of the stearyl hydrazide heparin derivative was collected, dialyzed, and freeze-dried.

*Purification of the Stearyl Hydrazide Heparin Derivative*—The stearyl hydrazide heparin sodium salt was dissolved in 16% aqueous sodium chloride (1 mL), and methanol (8 mL) was added to precipitate the stearyl hydrazide heparin. This process was repeated three times to remove any unreacted stearyl hydrazide from the product. Finally, the residue of stearyl hydrazide heparin derivative was reconstituted in 1 mL of water, dialyzed against water using a 3500 MWCO membrane, and freeze-dried. The recovered yield was 77%.

*$^1H$-NMR Analysis of the Heparin Hydrazide Derivatives*—All the hydrazide derivatives of heparin were prepared for NMR analysis as previously described and showed the following spectral characteristics: residue IdoAp2S, δ 5.20 (H-1), 4.45 (H-2), 4.25 (H-3), 4.10 (H-4); residue GlcNp2S, δ 5.50 (H-1), 3.30 (H-2), 3.70 (H-3), 3.90 (H-4), 4.15 (H-5); residue GlcAp, δ 5.60 (H-1); residue GlcNpAc, δ 2.10 (s, $COCH_3$); the methylene and methyl signals of various heparin hydrazide derivatives appeared at δ 0.97 [br t, $CH_3(CH_2)_nCO$], 1.12–1.25 [br m, $CH_3(CH_2)_nCO$], 1.60 [br t, $CH_3(CH_2)_nCH_2CO$].

*Hydrogenation of the $C_{12}$ Heparin Hydrazide Derivative*—Hydrogenation of the azo bond in the laurate hydrazide derivative of heparin was carried out in a Parr hydrogenator at a pressure of 47 psi using 10 mg of palladium on activated charcoal as a catalyst. The laurate hydrazide derivative of heparin (12 mg) was dissolved in 8 mL of distilled water and placed in a hydrogenation flask, tightly fitted within the instrument, and the flask was flushed with hydrogen gas and then evacuated under vacuum three times to remove all the air present within the flask. The flask was then filled with hydrogen gas for 48 h under a pressure of 47 psi after which the reaction product was filtered through a 0.45-μm filter and the aqueous solution was collected and freeze-dried. The yield of the reduced laurate hydrazide derivative of heparin was 88%. The hydrogenation of the azo bond in this derivative was

confirmed by measuring the reactive amino group formed by using *N*-4-[6-(dimethylamino)-2-benzofuranyl]phenyl (NDBP) isothiocyanate hydrochloride. The reduced form of the heparin derivative was reacted with the fluorescent NDBP isothiocyanate in formamide (using the method described above). Fluorescent labeling of the reduced heparin derivative was confirmed by polyacrylamide gel electrophoresis using an ultraviolet transilluminator.[14]

*Reaction of the Reduced Laurate Hydrazide Derivative of Heparin with Octadecyl Isocyanate*—The tetrabutylammonium salt of the reduced laurate hydrazide derivative of heparin was prepared from its sodium salt (6.0 mg) by Dowex 50 ($Bu_4N^+$ form) cation-exchange resin. The tetrabutylammonium salt of reduced laurate hydrazide derivative of heparin (14 mg in 25 mL dry methylene chloride) was refluxed for 24 h with 50 μL of octadecyl isocyanate. The methylene chloride was evaporated, the residue dissolved in 5 mL distilled water, and the product was exchanged into the sodium salt using Dowex 50 ($Na^+$ form). The product was purified by dissolution in 16% sodium chloride and repeated precipitation from methanol (as described above). The yield was 76%. The modified heparin derivative contained a $C_{12}$ and $C_{18}$ fatty acid chain attached through a urea type of linkage. $^1H$ NMR of the product showed a significantly increased signal intensity around $\delta$ 1.23 ppm when compared to that of the reduced laurate hydrazide derivative of heparin, corresponding to the addition of an octadecyl ($C_{18}$) substituent.

*Measurement of Partition Coefficients in Octanol–Phosphate Buffer*—The heparin derivative (10 mg) was dissolved in 1 mL of 5 mM sodium phosphate buffer, pH 7.0. This solution was added to 10 mL of octanol (previously saturated with the same buffer) and placed in a sealed tube, and the resulting two-phase system was shaken for 48 h at 30 °C. The octanol layer was carefully recovered (7.5 mL of the 10 mL) without disturbing the aqueous layer. The octanol solution was extracted three times with 10 volumes of water. The water extracts were combined and freeze-dried, and the heparin content was measured by a carbazole assay.[11]

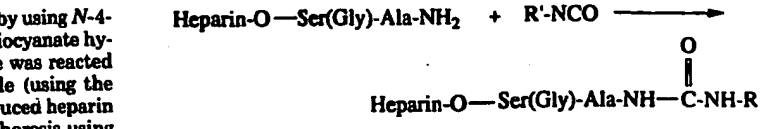
*Determination of Binding Affinity to Phenyl-Sepharose CL-4B*—Heparin or a heparin derivative (50 μg) was dissolved in 100 μL each of water and 2.0 M (pH 3.5), 3.0 M (pH 3.4), and 3.8 M (pH 3.3) ammonium sulfate solutions. Each sample was added to 100 μL of phenyl-Sepharose equilibrated with the same buffer. After incubation overnight at 4 °C, the supernatant from each vial was recovered by centrifugation (2 min at 11000*g*) and the concentration of heparin was determined by the carbazole assay.[11] The percentage of free heparin was measured and the percentage of heparin bound to phenyl-Sepharose in each sample was then calculated.

*Biological Assays*—The USP assay was performed against the USP K2 heparin standard. The antifactor Xa activity was determined by amidolytic assay using normal human plasma as a source of antithrombin III, bovine factor Xa, and chromogenic Xa substrate. The antifactor Xa activity of the heparin derivatives was calculated from a standard curve prepared using the USP K2 heparin standard.

## Results and Discussion

Heparin is biosynthesized in mast cells as proteoglycan (MW ~1 million). Because of the presence of endoglycuronidase and proteases in the tissue, heparin is processed into a glycosaminoglycan of reduced molecular weight (MW 5000–20 000).[1] Approximately 10% of these glycosaminoglycan chains contain a small portion of the core protein. These chains are called peptidoglycan heparin. All the amino groups on the heparin polysaccharide are either *N*-acetylated or *N*-sulfated.[13] Thus the free amino groups on the core peptide are the only groups free to react with an isocyanate. Peptidoglycan heparin also differs from glycosaminoglycan heparin in that the core linkage region contains a xylose and two galactose residues and thus has a very low level of sulfation.

The coupling reaction of peptidoglycan heparin and octyl isocyante is shown in Scheme 1. Using similar chemistry, fluorescent tags have been successfully introduced into peptidoglycan heparin and used as a method to isolate the sequence heparin's linkage region.[14] Phenyl-Sepharose CL-4B was used for fractionation based on the expected hydrophobicity of octyl-peptidoglycan heparin.[9,10] An octyl group, introduced onto the core peptide attached to the undersulfated linkage region, sufficiently increased hydrophobicity, enhancing the interacti n




**Scheme 1**—Synthetic scheme for the coupling f peptidoglycan heparin to alkyl isocyanates. R is a $C_8$ or $C_{18}$ alkyl chain.

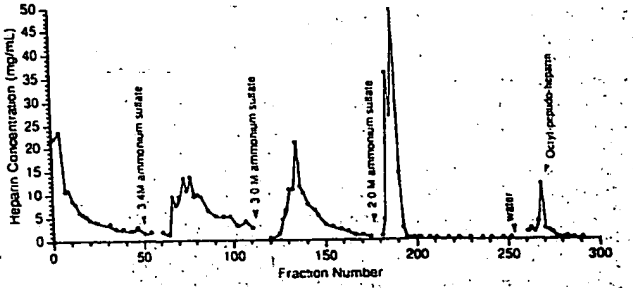



**Figure 1**—Enrichment of octyl-peptidoglycan heparin using a phenyl-Sepharose chromatography column. The octyl isocyanate-reacted heparin (1 g) was loaded on phenyl-Sepharose column (5 × 25 cm) which was pre-equilibrated with 3.8 M ammonium sulfate. The column was then washed with 3.8 M (pH 3.3), 3.4 M (pH 3.35), 3.0 M (pH 3.4), and 2.0 M (pH 3.5) ammonium sulfate solutions and the octyl-peptidoglycan heparin was eluted from the column by using water. The separation was performed at 4 °C. The concentration of heparin in the eluent was determined by a carbazole assay.[10]

**Table 1—Yield and Activity of Heparin Derivatives**

| | Isolated Yield (%) | USP Activity (IU/mg) | Anti Xa (IU/mg) |
|---|---|---|---|
| Heparin | na[a] | 158 | 158[b] |
| Raw heparin | na[a] | 150 | 145[b] |
| Octyl-peptidoglycan heparin | 7 | 107 | 88 |
| Octadecyl-peptidoglycan heparin | 7 | 116 | 56 |
| Octyl heparin | 36 | 183 | 171 |
| Capryl heparin | 40 | 182 | 159 |
| Lauryl heparin | 75 | 188 | 157 |
| Stearyl heparin | 76 | nd[c] | nd[c] |
| $C_{12}$,$C_{18}$-heparin | 76 | 57 | 48 |

[a] Not applicble. [b] Anti Xa activity and USP activity of heparin and raw heparin are considered identical. [c] Not determined.

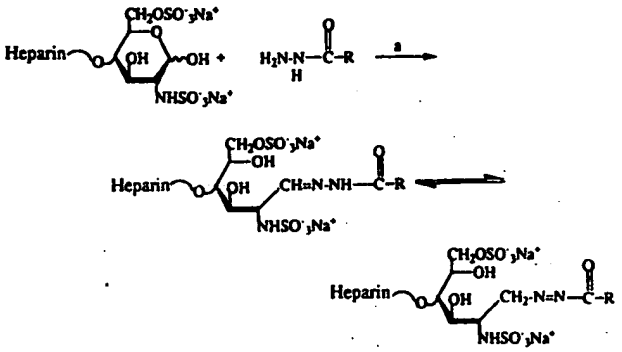
**Table 2—Comparison of Heparin Derivatives and Underivatized Heparin Binding Affinity to Phenyl-Sepharose**

| Binding Media (Ammonium Sulfate (M)) | Percentage Bound | | | |
|---|---|---|---|---|
| | Heparin | Octyl-Peptidoglycan | $C_{18}$ hydrazide | $C_{12}$,$C_{18}$ derivative |
| 3.8 | 68 | 96 | 97 | nd[a] |
| 3.0 | 46 | 98 | 83 | nd |
| 2.0 | 0 | 77 | 68 | nd |
| 0.0 | 0 | 48 | 49 | 53 |

[a] Not determined.

of octyl-peptidoglycan heparin with phenyl-Sepharose CL-4B. The purified octyl-peptidoglycan heparin was recovered from the phenyl-Sepharose column in the water fraction (Figure 1).

Octyl-peptidoglycan heparin was isolated in a yield of ca. 7% (w/w), based on raw heparin starting material, as determined by carbazole assay. This yield is equivalent to the isolation to ~70% of the peptidoglycan present in raw heparin. The presence of the octyl group in octyl-peptidoglycan heparin was confirmed using $^1H$ NMR. Bi logical assays indicated that octyl-pepti-



**Scheme 2**—Synthetic scheme for the coupling of acyl hydrazides with heparin. Step a is performed in formamide on the sodium salt of heparin when R is $C_8$, $C_{12}$, and $C_{14}$ and performed in methylene chloride on the tetrabutylammonium salt of heparin when R is $C_{18}$.

doglycan heparin had 107 IU/mg of USP activity and 88 IU/mg antifactor Xa activity (Table 1). These data show that the octyl-peptidoglycan heparin maintained about 75% of the anticoagulant activity of underivatized raw heparin. Thus, derivatization at the peptide core had a limited (if any) affect on the anticoagulant activity.

This octyl-peptidoglycan heparin shows a significant increase in its binding affinity to the phenyl-Sepharose column (Table 2). At highest salt concentration, 97% of octyl-peptidoglycan heparin binds to the phenyl-Sepharose column, while only 68% of underivatized heparin binds. Two hydrophobic groups, the octyl group and the peptide group, are present in octyl-peptidoglycan heparin. In addition, this derivative contains four unsulfated sugar residues in the linkage region. The linkage region structure of heparin is →4)-β-D-GlcAp(1→3)-β-D-Galp-(1→3)-β-D-Galp(1→4)-β-Xylp-1→O-Ser-(Gly), $AlaNH_2$[14] (GlcA, glucuronic acid; Gal, galactose; Xyl, xylose). The increased interaction of octyl-peptidoglycan heparin with phenyl-Sepharose is attributable to its enhanced hydrophobicity, the result of the hydrophobic functional groups present. The peptide and uncharged linkage oligosaccharide also provide a less charged context for the expression of the full hydrophobic character of the $C_8$ octyl substituent. Raw heparin (containing only the core peptide and unsulfated linkage region), however, binds to phenyl-Sepharose with approximately the same avidity as heparin (Table 2).

Attempts to measure the octanol–phosphate buffer partition coefficient of octyl-peptidoglycan heparin failed. No octyl-peptidoglycan heparin was detected in the octanol layer presumably due to the insufficient hydrophobicity of the heparin derivative. Octadecyl ($C_{18}$)-peptidoglycan heparin was prepared to examine whether increased hydrophobicity could be achieved by extending the fatty chain by an additional 10 methylene units. Octadecyl-peptidoglycan heparin was recovered from raw heparin in a reduced yield of ~7%, presumably due to the increased number of steps required when using chloroform as a solvent. It had high USP and antifactor Xa activities of 116 and 56 IU/mg, respectively (Table 1). This derivative bound tightly to phenyl-Sepharose, eluting only in the presence of 35% ethanol. The octadecyl-peptidoglycan heparin was still insufficiently hydrophobic to permit the measurement of an octanol-phosphate buffer partition coefficient.

In addition to being insufficiently hydrophobic, the derivatives of peptidoglycan heparin have a second, major disadvantage. Since peptidoglycan heparin only represents ~10% of raw heparin, the theoretical product yield is only 10% (an actual product yield of 7% was obtained). A different approach was required, if the goal to produce a hydrophobic heparin for

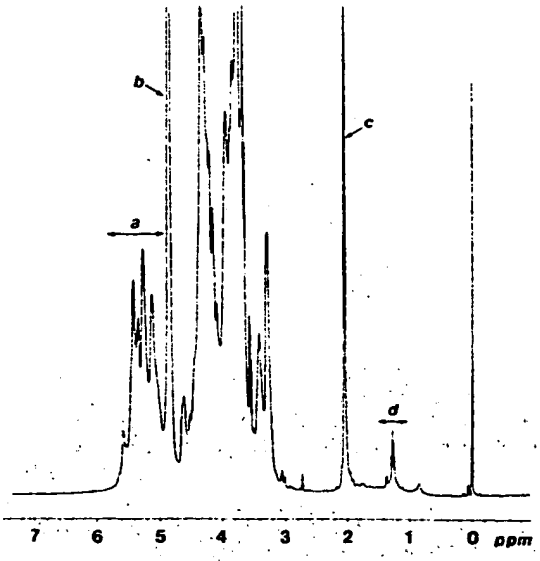



**Figure 2**—The 500-MHz $^1$H-NMR spectrum of the ($C_{12}$) lauryl hydrazide derivative of heparin. The signals are labeled as follows: *a*, corresponding to the anomeric protons of heparin; *b*, corresponding to HO$^2$H; *c*, corresponding to *N*-acetyl ($CH_3$); and *d*, corresponding to the methylene signals of the lauryl chain.

pharmaceutical applications was to be realized. Acyl hydrazides are known to react at the reducing ends of carbohydrates to form hydrazone derivatives that are further stabilized on undergoing tautomerization.

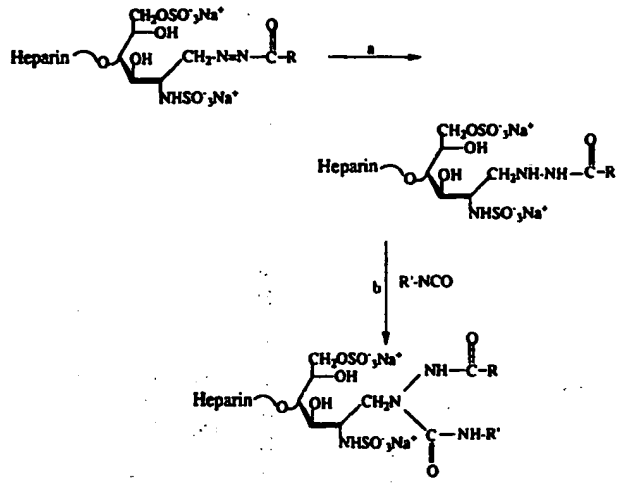
The coupling reaction of acyl hydrazide to the reducing end of heparin is shown in Scheme 2. A 10-fold molar excess of acyl hydrazide was used to drive this reaction to completion. The formation and purity of the hydrazide derivatives of heparin derivatives were confirmed by $^1$H-NMR spectroscopy (Figure 2) by comparing the signals of the alkyl chain at δ 1.2–1.8 with the signals corresponding to the anomeric protons of the heparin chains at δ 5.00–5.55 (eq 1 and 2).

$$\text{area ratio} = \frac{[\text{area of peak at 5.00–5.55 ppm (anomeric protons)}]}{[\text{area of peak at 1.2–1.8 ppm (methylene protons)}]} \quad (1)$$

$$\text{purity of heparin derivative} = \text{area ratio} \times \frac{[\text{no. of methylene protons in alkyl group}]}{[\text{avg MW heparin}/\text{avg MW of a saccharide unit}]^*} \times 100\% \quad (2)$$

*The avg MW of heparin is 14 000; the avg MW of a saccharide unit is 300.

Since unreacted acyl hydrazide had been completely removed by methanol precipitation and exhaustive dialysis, the alkyl protons observed between 1.2 and 1.8 ppm in the $^1$H NMR correspond only to the modified heparin. The 500-MHz $^1$H-NMR spectrum clearly shows four anomeric peaks at ca. 5.0, 5.2, 5.35, and 5.5 ppm. The two predominant anomeric signals, also observed in the heparin starting material, are 5.2 and 5.5 ppm correspond to the H-1 of iduronic acid 2-sulfate and glucosamine 2,6-disulfate, respectively. The additional peaks at 5.0 and 5.35 ppm correspond to the H-1 of iduronic acid and glucosamine 2-sulfate, respectively. Although these residues are present in heparin, they are clearly enriched in the hydrophobic heparin derivatives. This suggests that some *O*-desulfation has occurred under the reaction conditions. Analysis of these heparin



**Scheme 3**—Synthetic scheme for the coupling of two alkyl chains to heparin. Step a is performed on the heparin hydrazide derivative when R is $C_{12}$ in 20% aqueous methanol with $H_2$ over Pd/C. Step b is performed in methylene chloride on the tetrabutylammonium salt of heparin when R is $C_{18}$.

derivatives for free amino groups,[15] which might be formed through *N*-desulfation, show none are present. Milder reaction conditions (i.e., reduced temperature) and/or the use of the tetrabutylammonium salt of heparin in less polar (i.e. methylene chloride) solvents are currently being examined to reduce *O*-desulfation.

The purity of the hydrazide derivatives of heparin determined by $^1$H-NMR analysis (Figure 2) ranged from 86 to 100%. A yield of between 36 and 76% was achieved with the derivatives containing longer alkyl chains, giving the higher yield (Table 1). This may be due to the fewer number of methanol precipitation steps required to remove the less water soluble large chain acyl hydrazides during product purification. The anticoagulant activity of these heparin derivatives was examined by USP assay (Table 1). The octyl, capryl, and lauryl hydrazide derivatives of heparin had activities of 183, 182, and 188 IU/mg, respectively. These heparin derivatives had ~115% of the activity of the starting heparin. This enhancement of anticoagulant activity was unexpected and cannot yet be adequately explained. The antifactor Xa activity of these heparins was also slightly increased. The increased activity of these derivatives might be the result of the presence of a hydrophobic chain or a reduction in their *O*-sulfation. The hydrophobicity of these heparin derivatives was examined by binding studies using phenyl-Sepharose. These heparin derivatives did not show enhanced binding affinity when compared to unmodified heparin. This result suggests that a single small linear aliphatic chain of $C_8$, $C_{10}$, and $C_{12}$ attached directly to the highly negative, polyanionic heparin molecule is unable to enhance hydrophobicity to tightly interact with phenyl-Sepharose. Alternatively, this linear alkyl chain, in the context of the highly negative heparin, might be too hydrophobic, self-associating the "balling up" in the derivative, preventing its interaction with phenyl-Sepharose. To examine these hypotheses, a larger, stearyl ($C_{18}$) hydrazide derivative was also prepared and its hydrophobicity examined. A slightly different approach was required to prepare the stearyl ($C_{18}$) hydrazide derivative of heparin because of the limited solubility of stearyl hydrazide in polar solvents and the limited solubility of heparin in organic solvents. The tetrabutylammonium salt of heparin in methylene chloride reacted with stearyl hydrazide, affording the modified heparin carrying a $C_{18}$ hydrocarbon chain in 76% yield. The resulting stearyl ($C_{18}$) hydrazide derivative of heparin had approximately the same binding affinity for phenyl-Sepharose column as octyl-peptidoglycan heparin.

The modest enhancement of hydrophobicity obtained on placing a $C_{18}$ hydrocarbon chain on either heparin or peptidoglycan heparin suggests that the highly negative charge of the heparin polyanion (heparin of MW 14 000 has a charge of ca. 80–) dominates the physical properties of the conjugate. It appeared possible that the derivatives hydrophobicity might, however, be significantly enhanced by the use of two lipophilic hydrocarbon chains. A strategy to incorporate two hydrophobic chains was designed (Scheme 3). The azo bond of the laurate hydrazide derivative of heparin was first reduced by hydrogenation and the reactive nitrogen of the hydrazide group thus formed was reacted with the octadecyl isocyanate. The resulting derivative has two long fatty acid chains attached to heparin through a urea-type linkage. Integration of the methylene protons in the $^1$H-NMR spectrum confirmed the formation of the expected product. Binding studies showed that the $C_{12}$,$C_{18}$-heparin had slightly higher hydrophobicity then either octyl-peptidoglycan heparin or the stearyl ($C_{18}$) hydrazide derivative of heparin (Table 2). However, while the antifactor Xa activity (48 IU/mg) of this derivative was comparable to the other modified heparins, its USP activity (57 IU/mg) was markedly reduced (Table 1). Attempts to measure the octanol–buffer partition coefficient of this modified heparin containing two fatty chains still showed that it had no measurable partitioning into the octanol layer.

In conclusion, this paper describes new approaches for the preparation of hydrophobic heparin derivatives. The octyl- and octadecyl-peptidoglycan heparins, while having increased hydrophobicity as measured by binding to phenyl-Sepharose, show a modest reduction in anticoagulant activity and low recovered yields from the raw heparin starting material. The $C_8$, $C_{10}$, and $C_{12}$ hydrazide derivatives of heparin are each obtained in high yield and exhibit increased anticoagulant activity but do not show an enhancement of hydrophobicity as measured by interaction with phenyl-Sepharose. This result suggests that such a chain is insufficiently hydrophobic to enhance the binding of heparin to phenyl-Sepharose. The larger stearyl ($C_{18}$) hydrazide derivative of heparin has slightly enhanced hydrophobicity, comparable to the octyl-peptidoglycan heparin. This slight increase in hydrophobicity suggests that the context in which the fatty acid chain is placed (i.e., directly on the polyanion or on a peptide attached to a neutral tetrasaccharide to which the polyanion is attached) greatly influences the derivatives hydrophobicity. The $C_{12}$,$C_{18}$ double fatty acid chain derivative shows a slight increase in hydrophobicity (compared to $C_{18}$ heparin) and a slight decrease in anticoagulant activity and can be prepared in reasonable yields but requires a multistep synthetic approach. These results do not permit us to distinguish between the two hypotheses advanced for the lack of significant hydrophobicity of these derivatives. They do, however, suggest a promising new strategy for preparing heparin derivatives having modestly enhanced hydrophobicity that retain anticoagulant activity.

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